Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 4467–4469
Bioorganic & Medicinal Chemistry Letters

A chemical strategy to promote helical peptide–protein interactions involved in apoptosis

Dongxiang Liu,[a] Bin Yang,[a] Rong Cao[c] and Ziwei Huang[a,b,c,*,†]

[a]*Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*
[b]*Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*
[c]*Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

Received 26 May 2005; accepted 12 July 2005
Available online 30 August 2005

Abstract—Protein–protein interactions often involve secondary structural elements, such as helices. Protein–protein interactions within the Bcl-2 family are mediated by the helical BH3 domain of proapoptotic family members, such as Bad, Bak, Bax, or Bid. Here, we report that two 5-residue fragments located at the N- and C-termini of the 16-residue BH3 domain of Bad, respectively, serve as affinity-enhancing motifs (AEMs) for the BH3 domain. When added to the BH3 domain derived from other proapoptotic proteins such as Bak, Bax, or Bid, these AEMs significantly increased the Bcl-2-binding affinity of these BH3 peptides by promoting the helical structure. This finding may point to a new strategy for studying and mimicking helical peptide–protein interactions involved in apoptosis.

Protein–protein interactions play an important role in a wide variety of physiological and pathological processes. The inhibition or promotion of these interactions, either by small molecules such as synthetic peptides or by nonpeptidic organic compounds, is of great interest for understanding the mechanism of biological recognition and developing novel therapeutic agents. In this regard, much progress has been made in recent years.[1,2] This type of chemical research on protein–protein interactions is becoming increasingly important, especially in the post-genomics era, in which small molecular regulators of protein–protein interactions can be used to study the function of new proteins uncovered by genomics research efforts.

A wide array of protein–protein interactions are involved in the process of apoptosis or programmed cell death. The chemical biology of apoptosis, which aims to understand the chemical basis of how cells die and to develop intervening strategies for this process when dysregulated in human diseases, is a rapidly growing area of research.[3,4] As key regulators of apoptosis, Bcl-2 and related Bcl-2 family member proteins have been the subject of intensive chemical, biological, clinical, and structural research. The Bcl-2 family includes a growing list of proteins that can either inhibit apoptosis (antiapoptotic), such as Bcl-2 and Bcl-x_L, or promote apoptosis (proapoptotic), such as Bak, Bax, Bid, and Bad. Despite the opposite functions of these anti- and proapoptotic proteins,[3] they share some common features, such as the BH1-4 domains conserved in their amino acid sequences. In general, the BH3 domain is shared and used by all proapoptotic Bcl-2 family members as a key element to bind and counteract antiapoptotic proteins. NMR studies have shown that a 16-residue peptide derived from Bak BH3 domain bound to Bcl-x_L in a helical conformation.[5] Synthetic peptides and small molecules that mimic these proapoptotic BH3 domains have been shown to induce apoptosis of cancer cells, thus acting as a new class of anticancer agents for a wide variety of cancers in which Bcl-2 or related proteins are implicated.[3]

We are interested in knowing more about the chemical mechanism of the interaction between BH3 peptides and Bcl-2 protein as a model for studying protein–protein interactions involving helical domains. One important challenge for using peptides or peptide-mimicking compounds to study or inhibit large protein–protein complexes is the lack of high protein binding

Keywords: Bcl-2; BH3; AEM.
* Corresponding author. Tel.: +1 858 713 9928; fax: +1 858 713 9927; e-mail: ziweihuang@burnham.org
† Present address: The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA.

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.07.031

affinity of synthetic peptides or small molecule mimics. In this study, we wanted to explore new strategies to enhance peptide–protein interactions using BH3 peptides and Bcl-2 protein as a test example. We noticed the very high Bcl-x_L- and Bcl-2-binding affinity, which is probably the highest among natural BH3 peptides of the same or shorter length and nonpeptidic compounds that mimic the BH3 domain, shown by peptides derived from Bad containing the 16-residue BH3 core sequence plus several additional residues at both its N- and C-termini.[6–8] Interestingly, the 16-residue Bad BH3 core peptide showed very low or no binding to Bcl-2 (Table 1) or Bcl-x_L.[6] Thus, it is apparent that the addition of N- and C-terminal segments of 4–5 residues led to this substantial increase in binding affinity.

In this study, we hypothesize that these N- and C-terminal sequences adjacent to the Bad BH3 core domain are affinity enhancing segments and propose that they are designated as N-AEM (affinity enhancing motif) and C-AEM, respectively. The amino acid sequences for N-AEM and C-AEM are NLWAA and SFKGL, respectively. To investigate the mechanism of action of these AEMs, we first posed the question as to whether N-AEM or C-AEM or both are needed for the affinity enhancement. Bad BH3 peptides containing the addition of N-AEM (N-AEM-Bad BH3) or C-AEM (Bad BH3-C-AEM) alone were synthesized (Table 1). Using a fluorescene polarization (FP)-based competitive binding assay,[9] we tested the Bcl-2 binding of these peptides as compared to Bad BH3 (the 16-residue core) and Bad BH3-26 (the 26-residue longer peptide containing both N-AEM and C-AEM). While N-AEM-Bad BH3 and Bad BH3-C-AEM showed increased binding affinity as compared to the inactive Bad BH3, both of them were still much less potent than Bad BH3-26 (Table 1). This demonstrates that both N-AEM and C-AEM are needed and together lead to a drastic enhancement in affinity. To ask the question whether any key residue(s) in these AEMs is important for the observed affinity enhancing activity, four mutant peptide analogs containing amino acid replacements at various positions of the N-AEM or C-AEM of the peptide were synthesized and tested. Except for the Phe → Ala mutation at the C-AEM that led to a ~4-fold reduction in affinity, the rest of the mutations did not have any significant effect on binding. These results suggest that these AEMs do not make any direct and major contribution to binding but rather likely serve as motifs to enhance the affinity of the BH3 core. Also supporting this argument was the finding that the individual N-AEM or C-AEM peptide sequence alone did not show any binding to Bcl-2 (data not shown). Our results are consistent with the study by Petros et al., who also found that these sequences did not contain key binding determinants contributing to significant interaction energy with Bcl-x_L.[6]

We tested whether the AEMs derived from Bad can be applied to enhance the Bcl-2-binding affinity of BH3 peptides derived from other proapoptotic proteins. The AEMs were added to BH3 core peptides derived from Bak, Bax, and Bid (Table 1). A significant increase in Bcl-2 binding affinity was seen in all three cases when the AEMs were applied (Table 1 and Fig. 1). While

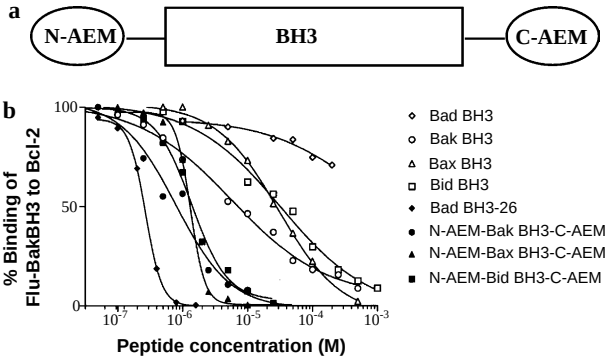

Figure 1. (a) AEM motifs are attached to the N- and C-termini of the BH3 domain peptide; (b) The Bcl-2-binding affinity of the BH3 domain peptides.

Table 1. Bcl-2-binding affinity of Bcl-2 family protein BH3 peptides and their analogs

BH3 domain peptides	Sequence	IC_{50} (μM)	% Helix
Bad BH3-26	NLWAAQRYGRELRRMSDEFEGSFKGL	0.30	13.1[a]
Bad BH3	QRYGRELRRMSDEFEG	N/A	14.1[a]
N-AEM-Bad BH3	NLWAAQRYGRELRRMSDEFEG	5.60	12.0[a]
Bad BH3-C-AEM	QRYGRELRRMSDEFEGSFKGL	17.50	13.4[a]
Bad BH3-26 (R2)	N**R**WAAQRYGRELRRMSDEFEGSFKGL	0.49	13.9[a]
Bad BH3-26 (A3)	NL**A**AAQRYGRELRRMSDEFEGSFKGL	0.41	13.8[a]
Bad BH3-26 (A22)	NLWAAQRYGRELRRMSDEFEG**A**FKGL	0.80	14.0[a]
Bad BH3-26 (A23)	NLWAAQRYGRELRRMSDEFEGS**A**KGL	1.36	13.4[a]
Bak BH3	GQVGRQLAIIGDDINR	8.36	6.1/7.3/14.6/21.8[b]
Bak BH3-26	PSSTMGQVGRQLAIIGDDINRRYDSE	3.03	7.3/8.9/25.2/37.7[b]
N-AEM-Bak BH3-C-AEM	NLWAAGQVGRQLAIIGDDINRSFKGL	0.65	8.1/11.4/56.7/67.7[b]
Bax BH3	KKLSECLKRIGDELDS	29.07	6.2/9.5/27.9/31.8[b]
Bax BH3-26	QDASTKKLSECLKRIGDELDSNMELQ	2.88	10.0/12.9/47.3/55.1[b]
N-AEM-Bax BH3-C-AEM	NLWAAKKLSECLKRIGDELDSSFKGL	1.33	18.9/32.9/65.5/72.8[b]
Bid BH3	RNIARHLAQVGDSMDR	22.34	5.8/8.6/18.6/32.6[b]
Bid BH3-26	QEDIIRNIARHLAQVGDSMDRSIPPG	14.08	9.8[a]
N-AEM-Bid BH3-C-AEM	NLWAARNIARHLAQVGDSMDRSFKGL	1.56	8.6/12.5/53.4/69.3[b]

[a] CD spectra were acquired in 10% TFE.
[b] CD spectra were acquired in 0%, 10%, 30%, 50% TFE.

the BH3 core peptides derived from these different proapoptotic proteins have different sequences and binding potency, affinity enhancement by the AEMs was notably consistent and significant, which suggests that the affinity enhancing effect of the AEMs might be generally applicable. To examine whether this observed affinity enhancement effect is dependent on the specific sequence of the AEMs, the AEMs in the above-described peptides were substituted by native sequences of Bak, Bax, or Bid at the corresponding segments. These native motifs derived from Bak, Bax, or Bid have a much less affinity enhancing effect than the AEMs derived from Bad. These results suggest that the AEMs found in Bad may represent highly optimized motifs for promoting the binding interaction of BH3 domain with antiapoptotic proteins of the Bcl-2 family.

To investigate the mechanism of action of the AEMs in enhancing BH3 peptide binding to Bcl-2, we studied the structures of these various BH3 peptides with or without the AEMs by using circular dichroism (CD) spectroscopy.[9] Whereas in aqueous solution or low (10%) TFE all of these peptides studied are unstructured with low (~6–14%) helical content, a substantial increase in helical structure was observed in higher (30% or 50%) TFE for AEM-containing peptides (Table 1). As shown in N-AEM-Bak BH3-C-AEM, N-AEM-Bax BH3-C-AEM, and N-AEM-Bid BH3-C-AEM where the AEMs were incorporated into these various BH3 peptides, a significant helical structure (53–72% helical content) was found. The helix-promoting ability of AEMs from Bad is sequence specific, as the corresponding native sequences from Bak and Bax, which were chosen as representatives to be studied here, have the helix promoting effect not as strong as the AEMs from Bad. The difference in the ability to promote helix stability of these native sequences versus the AEMs from Bad seems to be in correlation with different binding enhancing effects. Taken together, these results suggest that AEMs from Bad act to stabilize the helical structure of the central BH3 peptide core, thus indirectly enhancing the helical peptide core binding for the Bcl-2 protein.

The discovery of AEMs adjacent to the BH3 core may have implications in understanding the mechanism of proapoptotic Bcl-2 family members, especially the so-called BH3-only members that are thought to act mainly by binding to antiapoptotic proteins via the BH3 domain. In the past, the 16-residue BH3 core sequence conserved among both proapoptotic and antiapoptotic proteins had been the subject of many structure–function studies. It might be worth investigating the role and mechanism of the AEMs found in Bad and the corresponding segments in other proteins in the function and interaction of these proteins. These motifs are non-conserved among Bcl-2 family proteins. Whether such sequence variations can be linked to the difference in affinity and selectivity observed in different Bcl-2-related protein–protein interactions remains to be addressed. The discovery of AEMs may also aid in the development of highly potent antagonists of Bcl-2 or Bcl-x_L. It has been shown by a number of recent studies that small molecule antagonists of Bcl-2 and/or Bcl-x_L are promising anticancer agents 3. These antagonists exert their biological activity by mimicking the BH3 core domain in the interaction with the receptor. A potential limitation of these small molecules is that they have a relatively lower binding affinity when compared to native proteins or peptides. The findings reported here about the AEMs suggest two new strategies to improve the binding affinity of small molecule antagonists of Bcl-2 and/or Bcl-x_L. The first is the addition of the AEMs to the molecular templates to generate AEM peptide–small molecule hybrids. This would be similar to what is described in this study for the AEM containing BH3 peptides. The second involves the conjugation of not the AEM peptides themselves but small molecules capable of mimicking the effect of the AEMs.

As helical domain–protein interactions are widely found in many biological systems, it remains to be seen whether the AEMs consisting of short amino acid sequences near the helical core binding domain like those observed in this study exist in other proteins. If such motifs are found, they may be used for generating high affinity protein-binding peptides employing a similar strategy as reported here. Such small and highly effective affinity enhancement templates are amenable for chemical synthesis and readily applicable to small molecule ligands.

Acknowledgments

This work was supported by grants from the National Institutes of Health and the American Cancer Society.

References and notes

1. Peczuh, M. W.; Hamilton, A. D. *Chem. Rev.* **2000**, *100*, 2479.
2. Cochran, A. G. *Curr. Opin. Chem. Biol.* **2001**, *5*, 654.
3. Huang, Z. *Chem. Biol.* **2002**, *9*, 1059.
4. www.nature.com/reviews/poster/apoptosis/index.html
5. Sattler, M.; Liang, H.; Nettesheim, D.; Meadows, R. P.; Harlan, J. E.; Eberstadt, M.; Yoon, H. S.; Shuker, S. B.; Chang, B. S.; Minn, A. J.; Thompson, C. B.; Fesik, S. W. *Science* **1997**, *275*, 983.
6. Petros, A. M.; Nettesheim, D. G.; Wang, Y.; Olejniczak, E. T.; Meadows, R. P.; Mack, J.; Swift, K.; Matayoshi, E. D.; Zhang, H.; Thompson, C. B.; Fesik, S. W. *Protein Sci.* **2000**, *9*, 2528.
7. Kelekar, A.; Chang, B. S.; Harlan, J. E.; Fesik, S. W.; Thompson, C. B. *Mol. Cell Biol.* **1997**, *17*, 7040.
8. Wang, J. L.; Zhang, Z. J.; Choksi, S.; Shan, S.; Lu, Z.; Croce, C. M.; Alnemri, E. S.; Korngold, R.; Huang, Z. *Cancer Res.* **2000**, *60*, 1498.
9. The in vitro Bcl-x_L-binding assay was performed on a LS-50 luminescence spectrometer (Perkin-Elmer). The Bak BH3 domain peptide labeled with 5-carboxyfluorescein (Flu-BakBH3) was used as the substrate ($k_D \approx 0.34$ μM). After 15 min of incubation, fluorescence polarization was measured at 530 nm with an excitation wavelength of 480 nm.